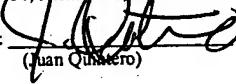


I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. **FV872312567US** on the date shown below in an envelope addressed to: MS RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: October 31, 2007

Signature:   
(Juan Quintero)

Docket No.: 29853/37702  
(PATENT)



## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:  
Shuyuan Zhang et al.

Application No.: 10/033,571

Confirmation No.: 97140

Filed: December 27, 2001

Art Unit: 1648

For: AN IMPROVED METHOD FOR THE  
PRODUCTION AND PURIFICATION OF  
ADENOVIRAL VECTORS

Examiner: B. P. Blumel

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

### DECLARATION OF PETER CLARKE, Ph.D. UNDER 37 CFR 1.132

I, Peter Clarke, PhD, hereby declare as follows:

1. I have been employed by Introgen Therapeutics Inc. for three years and my current position with this company is Vice President of Production and Technical Processes. Furthermore, I have over 25 years of experience in the biotechnology field working in both research and manufacturing. I have held positions at numerous biotechnology and pharmaceutical companies such as Director of Manufacturing at Chiron and Bayer Biological Products. In addition, my educational background includes a B.Sc. in biochemistry at Sheffield University in England, a Ph.D. in microbial physiology from the University of London, UK and a DIC in biochemistry from the Imperial College of Science and Technology in London, UK. A copy of my curriculum vitae is attached as Exhibit 1.

2. I understand that in reference to U.S. Patent Application Serial No. 10/033,571 (the '571 application). The Examiner has taken the position that a skilled person facing the technical problem of making pharmaceutically acceptable adenovirus composition would arrive at the process exemplified by claim 70 in view of the combined teachings of Shabram et al. (US Patent 5,837,520), Huyghe et al. (Human Gene Therapy, 1995), Kozak et al. (developments in Biological Standardization, 1996), Keay et al. (Biotechnology and

an adenovirus preparation having a level of contaminating nucleic acid similar to that claimed in the '571 case. However, it is my opinion that it is unlikely that the methods of Shabram would be sufficient to achieve the claimed level of purity (*i.e.*, a contaminating nucleic acid content of less than 400 pg per  $10^{10}$  pfu virus and greater than or equal to about 60 pg per  $10^{10}$  pfu virus).

3. Methods for preparing adenovirus according to Shabram call for enzymatically treating a cell lysate comprising a viral vector (*e.g.*, with benzonase) followed by further chromatographic purification (see, *e.g.*, column 2, lines 12-24). However, experiments from my laboratory have revealed that treatment of crude virus harvest (cell lysates) with benzonase results in adenoviral preparations that have extensive nucleic acid contamination. This is because Benzonase activity is significantly inhibited by the low pH (generally <7.0) of the cell lysate and also because of the presence of a large amount of proteases in the cell lysate. For Example, I would like to point to the examiner to page 10 of the Benzonase endonuclease product pamphlet (Exhibit 1). On page 10 of the pamphlet, figure 6 demonstrates that low pH dramatically reduces the activity of Benzonase endonuclease. As a result the contaminating nucleic acids in the cell lysates cannot be adequately degraded by the added Benzonase. Thus, the methods taught by Shabram would not enable a skilled artisan produce purified adenovirus that approaches the level of purity achievable by preferred methods according to the '571 application.

4. The Examiner alleges that Sastry et al. (Human Gene Therapy, 2004) provides evidence that benzonase could be used to reduce contaminating nucleic acid levels to a point commensurate with those recited in the '571 case. However, I have reviewed the Sastry reference and it does not provide evidence to support the Examiner's position. In particular, Sastry concerns benzonase treatment of lentiviral preparations. Careful review of Sastry indicates that, in addition to relating to a completely different class of virus (lentivirus vs. adenovirus), the virus preparations treated in Sastry are infected cell supernatants that have been subject to ultracentrifugation or ultrafiltration *prior* to benzonase treatment (see paragraph 4 on page 222 of Sastry). Thus, the preparations that were studied by Sastry were not cell lysates and the amount of cellular contaminants would be expected to be much lower. This is important for two reasons. First, the starting nucleic acid contamination level in the Sastry lentivirus preparations would likely be much lower than the contamination level in a

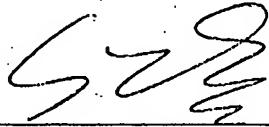
cell lysate comprising adenovirus. Secondly, the lower general level of contamination in a cell supernatant that has been subjected to ultracentrifugation or ultrafiltration (e.g., lower amounts of cellular proteins and lipids) is likely to have a less significant effect on benzonase activity as compared to the contaminants found in a adenovirus-infected cell lysate. Thus, in the case of the Sastry lentivirus preparations, initial nucleic acid contamination is lower and benzonase enzyme activity is higher. For these reasons, it is my opinion that the results from Sastry do not support the argument that adenovirus purification methods of Shabram could achieve the purity levels claimed in the '571 application merely by benzonase treatment.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any U.S. patent issued in this application.

Date:

29 Oct 07

By:

  
Shuyuan Zhang, Ph.D.

## **CURRICULUM VITA**

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## **EDUCATION**

University of Surrey, UK, 1990-1993

PhD in Animal Cell Technology, February 1993  
Thesis: Bubble Oxygenation of Both Low and High Density Animal Cell Cultures  
for Monoclonal Antibody Production

East China University of Science and Technology, Shanghai, China, 1984-1987

M.Sc. in Biochemical Engineering, February 1987  
Thesis: Mixing in Filamentous Fermentation Process

Tianjin University, Tianjin, China, 1980-1984

B.Sc. in Biochemistry, September 1984

## **WORK EXPERIENCE**

**Director, Analytical Service and Process Development, July, 2004-present**  
Introgen Therapeutics, Inc. Houston, TX  
Directing Process Development activities for adenoviral vector production and  
formulation processes  
Directing Analytical method development and testing for in process and final  
product samples

Interacting with Regulatory Agency for production process and product testing issues

Drafting Investigational New Drug (IND) submission document

Writing CTD regulatory submissions for FDA and EMEA

**Associate Director, Production and Process Development, January, 2002- June, 2004**

Introgen Therapeutics, Inc. Houston, TX

Directing Process Development and GMP Production activities for adenoviral vector production

Directing overall Process Validation efforts for CMC filing and BLA filing activities

**Associate Director, Process Development, June 1999-January, 2002**

Targeted Genetics Corp, Sharon Hill, PA

Directing the Process Development Department activities for both adenoviral and adeno-associated viral vector product process development and production.

Process Development involves cell culture, scale up to large bioreactors, downstream processing, tangential flow filtration for concentration and diafiltration, and chromatographic purification development and use of variety of analytical characterization methods. Production of the viral vectors using the developed processes under GLP and GMP conditions.

In charge of technology transfer and implementation in a Contract Manufacturing Organization for clinical production of viral vectors under GMP regulation

**Manager, Process Development, October 1995-June 1999**

Introgen Therapeutics, Inc., Houston, TX

Managed process development for upstream cell culture, downstream processing and chromatographic purification/characterization and formulation activities for retroviral and adenoviral vector products. Production of the vectors using developed processes

Actively involved in the collaboration with a large pharmaceutical company on adenoviral vector product development under GMP regulations

Key personnel in making production, purification and formulation process decisions

Actively participated in GMP production of adenoviral vector

**Research Scientist, Process Scale Up, February 1993- October 1995**

Genetic Therapy, Inc, Gaithersburg, MD

Responsible for process development, scale up and production of retroviral vectors from a variety of producer cells

Successfully developed large-scale retroviral vector production and purification process for clinical applications. The development resulted in the issuance of two US patent.

Large-scale production of clinical grade retroviral vectors

**Research Associate**, February 1987- January 1990

East China University of Science and Technology, Shanghai, China

#### **PROCESS DEVELOPMENT AND MANUFACTURING EXPERIENCE**

1. Successful development of a production process for retroviral vector production including upstream bioreactor design, downstream tangential flow filtration concentration and chromatographic purification and lyophilization.
2. Successful development of a microcarrier culture based production process for large-scale adenoviral vector production
3. Development of a chromatographic purification and TFF concentration process for adenoviral vectors
4. Development of a chromatographic purification and TFF concentration process for retroviral vectors
5. Development of serum-free, single cell suspension production process for adenoviral vectors
6. Actively involved in process harmonization activities with a major pharmaceutical company for adenoviral vector production
7. Successful development of a production and chromatographic purification process for adeno-associated viral vectors.
8. Involved in the development of novel liposome based non-viral gene delivery formulations
9. Have extensive experience and knowledge in process development and chromatography purification for the most commonly used gene delivery vehicles including: retroviral, adenoviral, adeno-associated viral and non-viral vectors
10. Manufacturing of retroviral, adenoviral and adeno-associated viral vectors in different organizations under GMP regulations.

#### **TECHNOLOGY TRANSFER EXPERIENCE**

Successfully managed the transfer of large-scale retroviral, adenoviral and adenoviral associated viral vector production and column purification process from Process Development Group to cGMP manufacturing for clinical production

Cost analysis of production and purification process and impact on marketable products

## **PROCESS VALIDATION**

Successfully validated large-scale adenoviral vector production and column purification process in a cGMP environment

Work closely with QC and QA for process validation and transfer to GMP production

Design and implement process validation plans for BLA CMC filing

## **cGMP EXPERIENCE**

Actively involved in the preparation of CMC document for FDA and EMEA submission for later stage clinical trials

Have good understanding of cGMP regulations and implementation.

Participated in the design of GMP facility and maintenance

Preparation and review of SOP and batch records

Managing the manufacturing of clinical grade viral products for gene therapy in a GMP compliant environment

Drafting Investigational New Drug (IND) submission document

## **RESEARCH AND DEVELOPMENT INTEREST AREA**

Cell culture and downstream processing and column purification for large-scale retroviral vector production for gene therapy

Cell culture and downstream process and column purification for large-scale adeno-associated viral vector production for gene therapy

Bioreactor for large-scale cell culture for AAV production from cell lines in serum-free media

Unit operation for downstream processing, tangential flow filtration

Chromatographic purification process for AAV

**Cell culture and downstream process and column purification for large-scale adenoviral vector production for gene therapy**

**Large-scale bioreactor cell culture process, stirred tank for serum-free suspension culture and Cellcube system for attached cells**

**Chromatographic purification process for adenoviral vectors**

**Formulation for viral vectors for gene therapy**

**Development of lyophilization formulation and technology that can be used for lyophilization of viral vectors for gene therapy**

**Development of stable, liquid formulation for adenoviral and AAV vectors**

**Non-viral gene delivery system**

**Development of stable liposome based gene delivery formulations  
Efficient gene delivery observed in animal studies**

**High cell density bioreactor system**

**Design of high cell density bioreactor system for viral vector production**

**Process optimization and cell metabolism in high cell density animal cell cultures**

**Optimization of high-density animal cell culture systems with a focus on oxygenation and nutrient metabolism**

## **AFFILIATIONS**

1. European Society of Animal Cell Technology (ESACT)
2. AAAS
3. American Chemical Society
4. American Institute of Chemical Engineers
5. The American Society of Gene Therapy
6. PDA
7. ISPE

## **PATENT**

1. Bioreactor for retroviral vector production, US 5,563,068
2. Purification of retroviral vector, US 5,661,022
3. Method for the purification and production of adenoviral vectors, US 6,194,191
4. Formulation of adenoviral vectors for gene therapy, US 6,689,600

5. Purified adenoviral compositions, US 6,726,907
6. Novel chromatographic purification process for AAV vectors, US patent pending

## **REFERENCES**

Available upon request

## **Exhibit 1**

# Benzonase® endonuclease

The Smart Solution for DNA Removal

Advancing Your Life Sciences –  
From Discovery to Launch™

EMD

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## Benzonase®

### endonuclease User References

18 Reference 1: Use of Benzonase® endonuclease in monoclonal antibody production  
Reference 2: Use of Benzonase® endonuclease in the downstream processing of recombinant FDH from *E.coli*  
Reference 3: Use of Benzonase® endonuclease to reduce cell clumping  
Reference 4: Use of Benzonase® endonuclease in adenovirus purification

## Benzonase®

### endonuclease

### Frequently Asked Questions

20-21 1) Which quality/quantity of Benzonase® endonuclease will be adequate for a certain application?  
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3) Do you offer immobilised Benzonase® endonuclease?  
4) Why is the Benzonase® endonuclease not working?  
5) I observe a loss of activity - why?  
6) At which step do I have to introduce Benzonase® endonuclease in my process?  
7) Is Benzonase® endonuclease free of protease activity?  
8) How do I remove Benzonase® endonuclease?  
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5) Detection of Benzonase® endonuclease - Benzonase® ELISA KIT II  
6) Removal of Benzonase® endonuclease

31 Literature references  
Key search words  
Internet links  
Patent and licence information  
Trademark information  
Disclaimer

How to meet the highest possible purity standards for biopharmaceuticals?

How to increase downstream processing yields?

# Benzonase® endonuclease

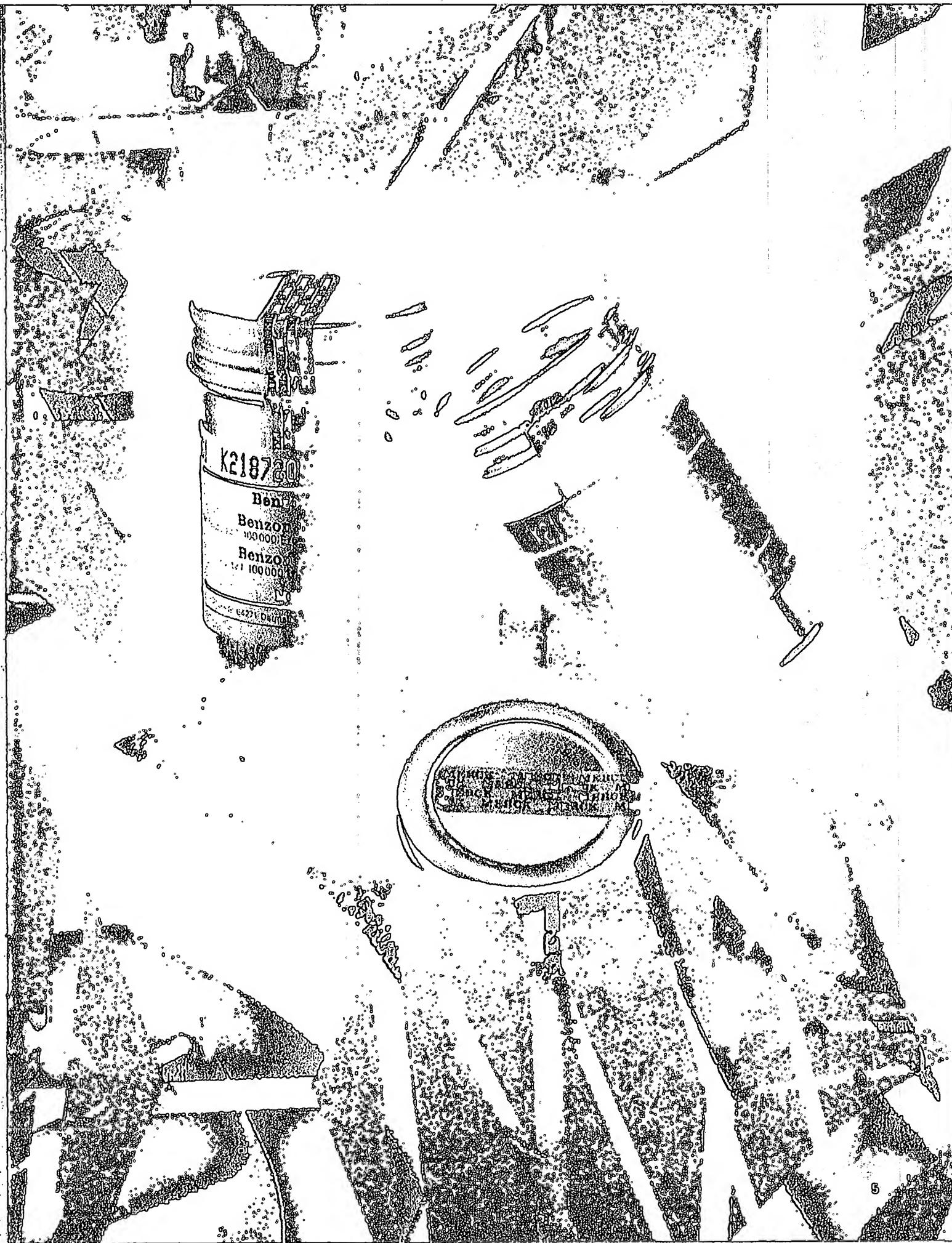


There is only one effective biochemical method to remove DNA and RNA, both in the laboratory and in industrial scale processes. It is called Benzonase® endonuclease.

Benzonase® endonuclease is a unique, genetically engineered endonuclease offering a variety of advantages over existing methods of nucleic acid removal.

Benzonase® endonuclease is manufactured and exclusively distributed by Merck KGaA Darmstadt, Germany and its associates.

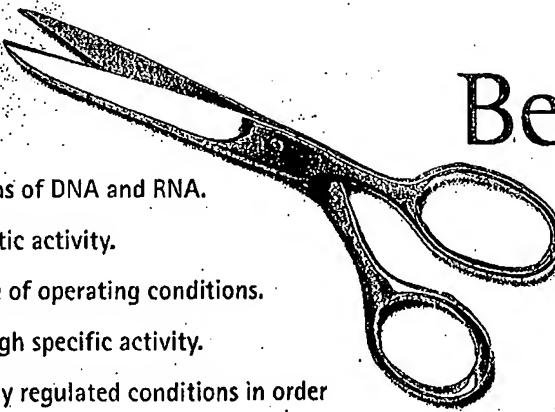
Benzonase® endonuclease has proven its value in the laboratory for over ten years and is currently successfully applied in various processes throughout the pharmaceutical and biotechnological industry.



# Benzonase® endonuclease

## Benzonase® endonuclease

- attacks and degrades all forms of DNA and RNA.
- is free of detectable proteolytic activity.
- is effective over a wide range of operating conditions.
- possesses an exceptionally high specific activity.
- is manufactured under strictly regulated conditions in order to meet industrial requirements for a reliable supply and consistent high quality.
- is ideal for a variety of applications including:
  - purification of proteins and other biologicals.
  - reduction of viscosity caused by nucleic acids.
  - sample preparation in electrophoresis and chromatography



## At a glance

Benzonase® endonuclease is a genetically engineered endonuclease produced in *Escherichia coli* strain W3110, a mutant of strain K12, which contains the proprietary pNUC1 production plasmid [1, 2]. This plasmid encodes an endonuclease normally expressed in *Serratia marcescens*. The production techniques used to manufacture Benzonase® endonuclease ensure a product of exceptionally high purity and activity. These techniques enable the enzyme to be supplied without measurable protease activity and without viral contaminants that can accompany enzymes isolated from natural sources.

To meet the widest possible range of processing and cost requirements, Benzonase® endonuclease is available in two different grades of purity

### Benzonase® endonuclease

#### Purity grade I (Ultra pure grade)

- more than 99% pure.

#### Benzonase® endonuclease

#### Purity grade II (Pure grade)

- more than 90% pure.

## Regulatory information

US FDA regulations are regarded as the world's most demanding standards of quality and purity for biopharmaceuticals. There are no explicit FDA regulations governing the production and use of excipients such as Benzonase® endonuclease. However, at Merck KGaA Darmstadt, Germany we place a high value on the safety of our products. For this reason Benzonase® endonuclease is produced under cGMP conditions (we are currently in the process of obtaining full cGMP validation\*). There also exists a DMF type II file for Benzonase® endonuclease at the US FDA (Reg. No. BBMF 5403). In addition to this Merck KGaA Darmstadt, Germany operates a quality management system according to DIN ISO 9001 and 14001.

### Auxiliary materials

The finished product does not contain additives of animal origin such as stabilisers like bovine serum, albumin or gelatine. Benzonase® endonuclease is supplied in 50 % glycerol solution to prevent freezing of the preparation. The glycerol is of synthetic origin.

During production of Benzonase® endonuclease the fermentation medium contains casamino acids from bovine milk. This milk originates from countries with no recorded BSE cases in locally bred animals since 1990 and is considered fit for human consumption. We can supply on demand full batch documentation. This documentation fulfils current US FDA requirements\*.

### Viral safety

We use a well defined bacterial expression System (*Escherichia coli* strain W3110 a K12 mutant) for the preparation of Benzonase® endonuclease. The risk of viral contamination is regarded as negligible.

### Microbiological safety

Each batch, with the exception of packages containing 10,000 units, is tested for the absence of aerobic bacteria, yeasts and moulds according to a modified EP method.

### Endotoxin testing

Each batch of Benzonase® endonuclease purity grade I is tested for endotoxins by an independent, accredited laboratory using the well known LAL-test. The total endotoxin level is below 0.25 EU per 1.000 units.

This field of regulatory information is constantly changing, for the latest updates please contact us at [processing@merck.de](mailto:processing@merck.de)

**C E R T I F I C A T E**

DOB GMBH  
Germany, Düsseldorf, Germany  
March 2004

Stadtwerke Straße 200  
D-44137 Düsseldorf  
with the experimental results as listed in the annex.

At the place  
Gronau (Westfalen), manufacturer of Benzonase® endonuclease,  
active chromosomal product and hybridomas

has implemented and maintains a  
Quality Management System.  
After evaluation it is noted, has confirmed that this  
Quality Management System fulfills the requirements  
of the following standard:

DIN EN ISO 9001:2000  
(October 2000 edition)

This certificate is valid until 2000-11-17  
Contract Registration No.: 002380 QM  
Ansbach am Main, Berlin 2002-11-18

R. K. S.  
Dr. med. A. Pfeifer

H. Böll  
DNA Lab. & Thermo

SAALAND DIREKT  
D-44137 Düsseldorf, Germany, Export-Vertrieb Benzonase®  
D-44137 Düsseldorf, Germany, Import-Vertrieb Benzonase®



# Product Description

## Product quality assurance: Using Benzonase® endonuclease to remove DNA/RNA

FDA guidelines for the manufacture of recombinant biologicals for therapeutic use demand that nucleic acid contamination should be limited to 10 pg per dose (in the end product) [3, 4]. However, this depends completely on the pharmaceutical indication area of the drug. Generally, nucleic acid contamination is measured by using appropriate DNA or RNA probes to detect hybridizable nucleic acids or by other sensitive DNA assays.

Benzonase® endonuclease, when used under appropriate reaction conditions, will degrade all nucleic acid sequences down to oligonucleotides of approximately 3 to 5 base pairs in length – which is significantly below the hybridization limit – enabling recombinant proteins to meet the FDA guidelines for nucleic acid contamination.

## Cell disintegration: Using Benzonase® endonuclease to reduce viscosity

The ability of Benzonase® endonuclease to rapidly hydrolyze nucleic acids makes the enzyme an ideal choice for reducing cell lysate viscosity – both in the research laboratory and the manufacturing plant.

Using Benzonase® endonuclease to reduce viscosity results in a number of benefits including:

- reduced processing time

- increased yield of protein products
- improved separation of pellet and supernatant in centrifugations
- facilitated filtration of solutions, especially ultrafiltration
- increased efficiency of chromatographic purification steps (e.g. in expanded bed adsorption) [5].

Benzonase® endonuclease may be used with all methods of cell lysis, including lysozyme treatment, freeze-thawing procedures and high-pressure homogenization. Although Benzonase® endonuclease may be added post lysis, it was shown that, when the same amount of Benzonase® endonuclease is added before lysis instead of afterwards:

- The amount of Benzonase® endonuclease required for nucleic acid hydrolysis can be reduced 50 to 200 times.
- Viscosity reduction will occur significantly faster.

## Particle processing: Using Benzonase® endonuclease to facilitate particle purification

It is well known that nucleic acids may adhere to cell-derived particles such as viruses or inclusion bodies [6-8]. This adhesion may interfere

with separation due to agglomeration, change in particle size or change in particle charge, resulting in a reduced product yield. Benzonase® endonuclease is well suited for reducing the nucleic acid load during purification, thus eliminating interferences and improving the yield.

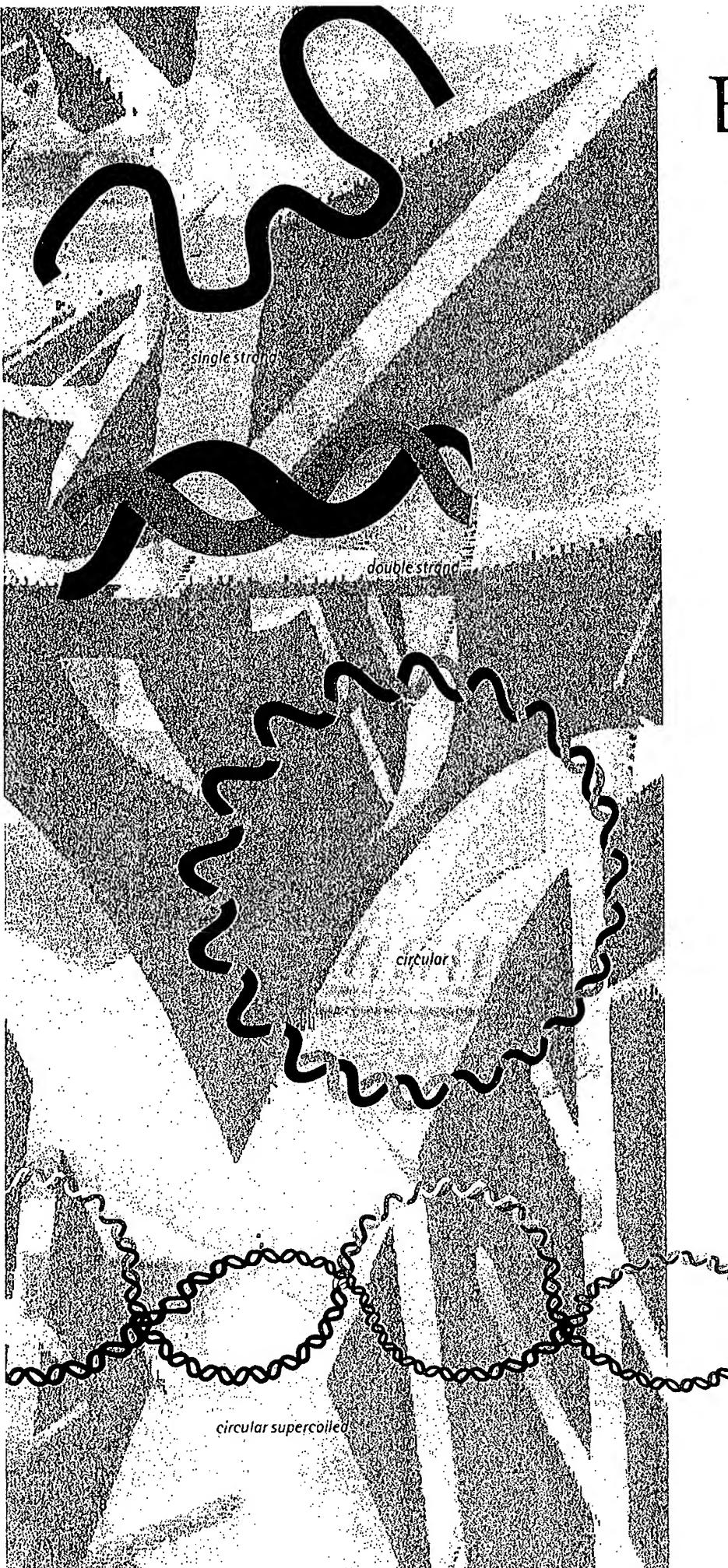
## Bioanalytical applications: Using Benzonase® endonuclease for sample preparation

Treating samples containing nucleic acids with Benzonase® endonuclease before they are used for analysis, e.g. ELISA, chromatography or two-dimensional electrophoresis (protein mapping) and footprint analysis [9] provides a number of benefits including:

- improved resolution, due to reduced interference from charged nucleic acid fragments present in the sample,
- increased recovery, due to reduced entrapment of product after reduction of sample viscosity.

These benefits are especially important when working with small sample volumes such as Mini-Gel electrophoresis or HPLC separations:

# Benzonase® endonuclease



Benzonase® endonuclease is a protein consisting of two subunits with a molecular weight of about 30 kD each. The protein has an isoelectric point (pI) at pH 6.85. It is functional between pH 6 and 10, and from 0°C to above 42°C. Mg<sup>2+</sup> (1-2 mM) is required for enzyme activity.

## Substrate specificity

Benzonase® endonuclease acts as an endonuclease that degrades both of DNA and RNA – whether single-stranded, double-stranded, linear, circular or supercoiled. No base preference is observed. As with all endonucleases, Benzonase® endonuclease hydrolyzes internal phosphodiester bonds present between the nucleotides. Upon complete digestion, all free nucleic acids present in solution are reduced to 5'-monophosphate-terminated oligonucleotides which are 3 to 8 bases in length.

## Activity

Specific activity for Benzonase® endonuclease is measured under standard assay conditions (see below). Minimum specific activity for Benzonase® endonuclease purity grade II (90 %) is  $1.0 \times 10^6$  units/mg protein. Minimum specific activity for Benzonase® endonuclease purity grade I (99 %) is  $1.1 \times 10^6$  units/mg protein.

# Enzyme Characteristics

## Temperature stability of Benzonase® endonuclease

The optimum temperature for the degradation of nucleic acids by Benzonase® endonuclease is 37°C. The enzyme is, however, effective over a temperature range of 0-42°C (see Fig. 1). The optimum storage temperature is -20°C to prevent loss of activity or freezing. The effect of storage at various temperatures is illustrated in Fig. 2.

We do not recommend repeated freeze/thaw cycles and storage at temperatures lower than -20°C, data can be supplied that shows no loss of activity during shipment.

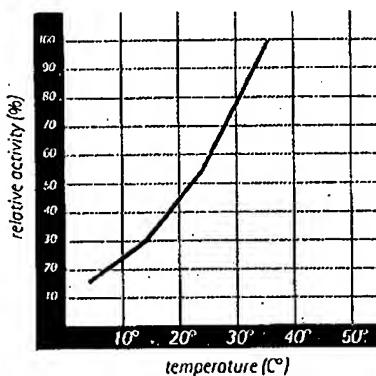
## Product purity

Analysis of Benzonase® endonuclease Pure Grade (purity grade II 90 %) by SDS-PAGE results in a dominant band corresponding to Benzonase® endonuclease. All other proteins present (<10 %) are derived entirely from *E.coli*.

Benzonase® endonuclease Ultra Pure Grade (purity grade I, 99 %) is produced by chromatographic purification of Benzonase® endonuclease Pure Grade. Analysis of Benzonase® endonuclease Ultra Pure Grade by SDS-PAGE results in a single band corresponding to Benzonase® endonuclease.

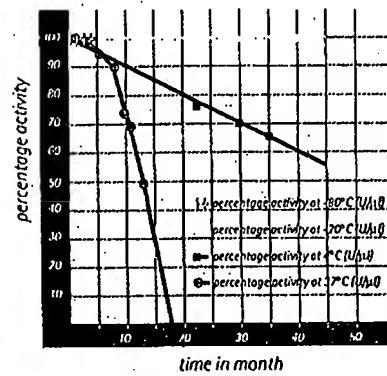
All other proteins present (<1%) are derived entirely from *E.coli*. The preparation does not contain any antimicrobial preservatives or protein stabilizers except glycerol (of synthetic origin). The solution has been filtered through a 0.2 µm filter.

Figure 1: Effect of temperature on Benzonase® endonuclease activity\*



\* Standard assay conditions  
(Substrate is DNA)

Figure 2: Effect of various storage temperatures on Benzonase® endonuclease activity over time.



30 minutes under standard assay conditions.

For a detailed description of the standard assay and additional sensitive nuclease assays see Appendix I.

## Protease activity

Benzonase® endonuclease is free of detectable protease activity, making the enzyme ideal for production processes in which high yields of biologically active proteins are desired. The absence of proteolytic activities is monitored by a highly sensitive and validated assay using a resorufin-labelled casein (Cat. No. 1.24852) as substrate (detailed instructions are available on request).

## Unit definition

A standard assay was developed to define the activity of Benzonase® endonuclease. The procedure is based on the measurement of changes in optical density that occur when oligonucleotides are released into solution during digestion of DNA with Benzonase® endonuclease. The assay is performed using excess substrate but other conditions are optimum. The rate of DNA degradation is measured by precipitation of undigested DNA using perchloric acid.

Based on this assay, one unit of Benzonase® endonuclease is defined as the amount of enzyme that causes a change in absorbance at 260 nm of 1.0 absorption units within 30 minutes. One unit of Benzonase® endonuclease also corresponds approximately to the amount of enzyme required to completely digest 37 µg of DNA in

# Benzonase® endonuclease

## Operating conditions

Benzonase® endonuclease retains its activity in a wide range of operating conditions, as specified in the following table and figures 3 to 6.

Table 1: Operating conditions for Benzonase® endonuclease

Condition	Optimal*	Effective**
Mg <sup>2+</sup> concentration	1 - 2 mM	1 - 10 mM
pH	8.0 - 9.2	6.0 - 10.0
Temperature	37 °C	0 - 42 °C
Dithiothreitol (DTT)	0 - 100 mM	> 100 mM
β-Mercaptoethanol	0 - 100 mM	> 100 mM
Monovalent cation concentration (Na <sup>+</sup> , K <sup>+</sup> , etc.)	0 - 20 mM	0 - 150 mM
PO <sub>4</sub> <sup>3-</sup> concentration	0 - 10 mM	0 - 100 mM

\* "Optimal" is defined as the operating range in which Benzonase® endonuclease retains ≥ 90% of its activity.

\*\* "Effective" is defined as the operating range in which Benzonase® endonuclease retains > 15% of its activity.

Figure 3: Effect of magnesium and manganese ion concentrations on Benzonase® endonuclease activity\*

A concentration of 1 to 2 mM Mg<sup>2+</sup> or Mn<sup>2+</sup> is essential for the activity of Benzonase® endonuclease. Mg<sup>2+</sup> is preferred because it enables the enzyme to reach its optimal level of activity. Ca<sup>2+</sup> and Sr<sup>2+</sup> do not effect the activity of the enzyme.

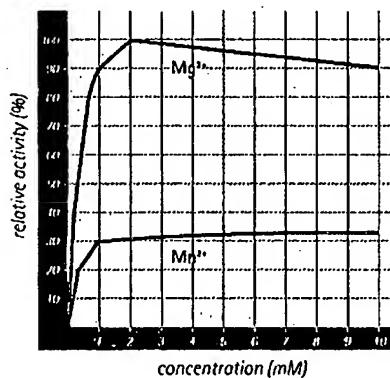


Figure 5: Effect of monovalent cations on Benzonase® endonuclease activity\*

The enzyme exhibits an identical response to Na<sup>+</sup> and K<sup>+</sup>. It is presumed that all other monovalent cations have a similar effect.

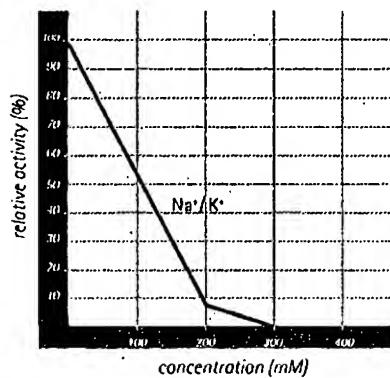


Figure 4: Effect of pH on Benzonase® endonuclease activity\*

The incubation buffers are 20 mM Tris, 20 mM histidine and 20 mM MES. Although not shown here, a slight buffer effect has been observed, indicating minimal changes in activity due to the effects of different buffers.

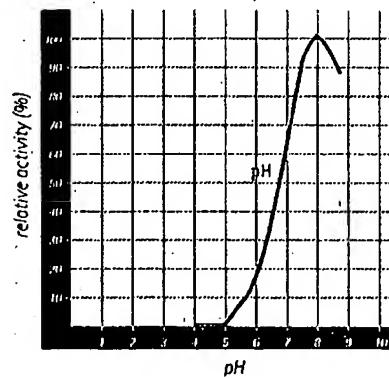
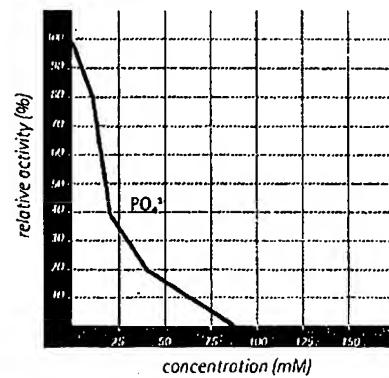


Figure 6: Effect of phosphate ion concentration on Benzonase® endonuclease activity\*

The incubation buffer is a Tris-phosphate buffer.



Effect of guanidine HCl, EDTA, and PMSF on Benzonase® endonuclease activity

Using the standard assay for Benzonase® endonuclease activity, it was shown that concentrations of guanidine HCl exceeding 100 mM completely inhibit the enzyme activity.

An EDTA concentration of 1 mM partially inhibits Benzonase® endonuclease. However, a concentration of 5 mM EDTA causes a >90% loss of enzyme activity by complexing the essential Mg<sup>2+</sup> ions. PMSF in a concentration of 1 mM does not inhibit Benzonase® endonuclease.

\* Standard assay conditions  
= 100% activity. Substrate is DNA.

\*\* Effects were determined by measuring the change in absorbance that occurs when oligonucleotides are released into solution.